Undergraduate Honors Research Thesis

Spectrin-Based Complex for Regulation of Signal Transducer and Activator of Transcription 3 Signaling and Heart Function

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Abstract

Maladaptive cardiac remodeling is an important step in the progression of heart failure and is characterized by changes in cardiac chamber size, structure, and performance induced by a constellation of cell- and tissue-level factors. While great strides have been made in identifying membrane receptors, signaling molecules, and transcription factors involved in the remodeling process, fundamental questions remain about the molecular pathways linking extracellular stress cues to cellular reprogramming underlying progression of disease. Signal Transducer and Activator of Transcription 3 (STAT3) is a multifunctional transcription factor which regulates expression of gene programs important for inflammation, cell survival, and hypertrophy. While STAT3 signaling modulates cardiomyocyte function in response to ischemia and biomechanical stress, the molecular pathways that coordinate STAT3 activity is unclear. We hypothesized that β_{IV}-spectrin, an actin associated cytoskeletal protein, coordinates a macromolecular complex with CaMKII and STAT3 to promote phosphorylation/activation of STAT3 at the cardiomyocyte submembrane. We believe that dysfunction of β_{IV}-spectrin will induce cardiac malfunction downstream of STAT3. To understand the interaction between STAT3 and β_{IV}-spectrin, novel mouse models expressing truncated β_{IV}-spectrin protein lacking the validated CaMKII binding domain (qv^{3J}), a cardiac-selective knockout (cKO) of β_{IV}-spectrin, and wildtype littermates were used. Cardiac function was assessed by echocardiography at baseline and post Transverse Aortic Constriction (TAC). Levels of total and phosphorylated STAT3 and CaMKII were assessed in whole heart lysates by western blot, pull-down, and co-immunoprecipitation assays to measure relevant protein concentrations and interactions. Permeabilized cardiomyocytes were immunostained for β_{IV}-spectrin, CaMKII, and STAT3 and protein localization was assessed by confocal microscopy to study the activation and localization of STAT3. β_{IV}-spectrin cKO mice showed decreased heart function while qv^{3J} mice displayed normal cardiac function at baseline

and post TAC, compared to wildtype. Mice lacking β_{IV}-spectrin/CaMKII/STAT3 interaction demonstrated abnormal STAT3 regulation and response to biomechanical stress. This study indicates the presence of a novel complex between β_{IV}-spectrin and STAT3, thereby regulating the localization of STAT3 in cardiomyocytes. Future studies will address the specific role of STAT3 in pathology associated with spectrin-deficiency.

Acknowledgements

I would like to thank my project advisor, Dr. Thomas Hund, PhD., (Department of Biomedical Engineering at The Ohio State University), for giving me the opportunity and privilege to conduct research in his lab at the Dorothy M. Davis Heart and Lung Research Institute. Dr. Hund has provided me with valuable insight and has guided me tremendously in the field of cardiac research.

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Table of Contents

Table of Figures

Introduction

Normal cardiac function depends on the ability of the heart to respond to various environmental stimuli (i.e. biomechanical/chemical stress, changes in pH, temperature, etc.). At the cellular level there are evolved pathways which facilitate the transduction of extracellular stimuli into electrical and/or chemical signals important for regulating physiological processes such as growth, proliferation, apoptosis, and electrical impulse generation and transmission for excitation and contraction. Central to these critical regulatory pathways are local signaling domains which exert a tight spatial and temporal control over the post-translational modification (i.e. phosphorylation, oxidation, glycosylation) [1].

Heart failure (HF) remains a major source of morbidity and mortality in the U.S. that is responsible for over 400,000 deaths each year and is affecting an estimated 5.7 million Americans with growing prevalence [2]. Maladaptive cardiac remodeling is an important step in the progression of HF and is characterized by changes in cardiac chamber size, structure, and performance induced by a constellation of cell- and tissue-level factors including hypertrophic growth, inflammation, fibrosis, and genetic reprogramming [3]. Although numerous membrane receptors, signaling molecules, and transcription factors involved in the remodeling process have been identified, specific molecular pathways linking extracellular stress cues to cellular reprogramming underlying progression of disease remain unclear.

β_{IV}-spectrin, an actin-associated cytoskeletal protein, helps maintain the structural integrity of cells and organizes local signaling domains for the regulation of ion channels and cell membrane excitability in cardiomyocytes [4]. Specifically, β_{IV}-spectrin targets the multifunctional Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) to ion channel substrates at the cardiomyocyte intercalated disc, a specialized membrane domain important for intercellular mechanical and electrical communication [3]. CaMKII is a serine/ threonine kinase that has a broad

substrate specificity and wide tissue distribution that phosphorylates a variety of different targets [5]. While a variety of membrane channels, receptors, and downstream signaling pathways have been implicated in mechanotransduction, the mechanistic link between specific biomechanical stimuli and cellular remodeling remains unknown.

Signal Transducer and Activator of Transcription 3 (STAT3), is a multifunctional, stress-activated transcription factor that regulates expression of gene programs important for cell growth, survival, inflammation, metabolism, and cytoprotection [6]. STAT3 plays a critical role in communication among cardiomyocytes, myocardial vasculature, and cardiac fibroblasts as well as modulation of cardiac microenvironment and its role in the regulation of cardiac inflammation [7]. Recent research efforts have shed light on the importance of STAT3 in proper functioning of heart, suggesting that STAT3 may serve as a novel therapeutic target for cardiac disease patients [6]. While STAT3 signaling modulates cardiomyocyte function in response to ischemia and biomechanical stress, the molecular pathways that coordinate STAT3 activity are unclear.

Tumor Necrosis Factor-alpha (TNF-α) is a cell signaling protein involved in systemic inflammation. Increased levels of TNF-α in circulation are known to cause death of cardiomyocytes and eventually lead to heart failure [8]. Previous findings have suggested that STAT3 is an upstream regulator of TNF-α and is able to regulate inflammatory responses of cardiomyocytes and is an important factor for protection and survival of cardiomyocytes *in vivo* [8]. Finding the optimal balance between STAT3 localization and activation may help develop novel therapies to treat heart failure, to protect the heart from unwanted drug-related side effects, and to protect it from oxidative and biomechanical stress.

We hypothesized that β_{IV}-spectrin coordinates a macromolecule complex with CaMKII and STAT3 to promote phosphorylation/activation of STAT3 at the cardiomyocyte submembrane.

We anticipate that dysfunction of β_{IV}-spectrin will induce cardiac malfunction downstream of STAT3 and that the novel complex will regulate maladaptive remodeling and arrhythmias in response to chronic stress.

To test our hypothesis, we used two genetically modified mice namely, *qv*^{3J}, and cardiac-selective knockout of β_{IV}-spectrin (β_{IV}-spectrin cKO). Wildtype mice have normal physiology and contain the full length of β_{IV}-spectrin along with CaMKII binding domains. β_{IV}-spectrin in the *qv*^{3J} mice lack the CaMKII binding domain (CTD) (Figure 1).

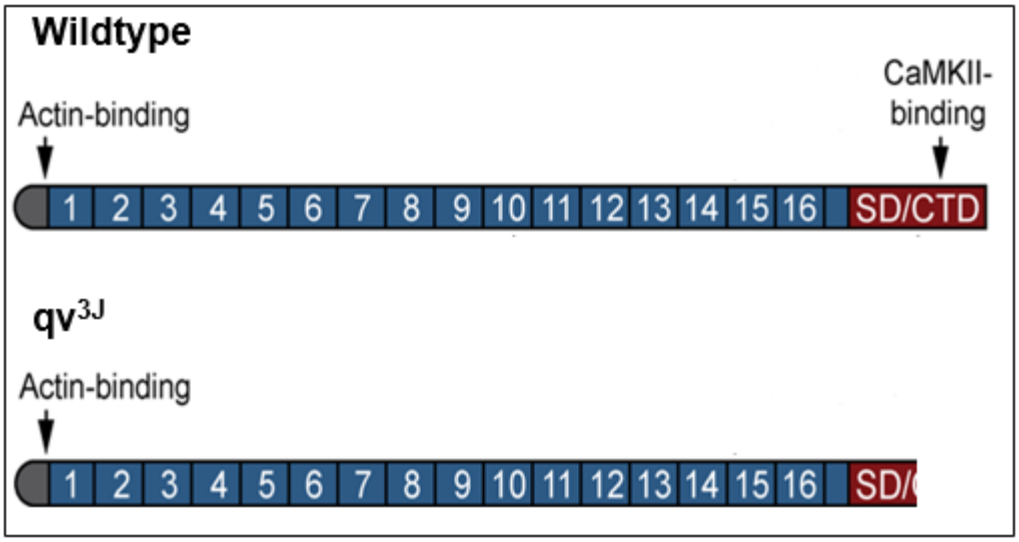

Figure 1: Protein sequence showing CamKII binding domains on the β_{IV}-spectrin protein. Full length of β_{IV}-spectrin is conserved in wildtype mice. *Qv*^{3J} mice lack the CaMKII binding domain.

Methodology

Experimental Mice Models

Adult mouse models that include novel inducible cardiac-selective knockout of β_{IV}-spectrin (β_{IV}-spectrin cKO), truncated β_{IV}-spectrin protein lacking the validated CaMKII binding domain (qv^{3J}), and wildtype (control) were used in this study. The β_{IV}-spectrin cKO mouse model was generated from a gene knockout targeting strategy. Specifically, a Cre/Lox system was used to remove exons 29 and 30 to generate the β_{IV}-spectrin cKO mouse model (Figure 2). Qv^{3J} mice were acquired from Jackson Labs [9].

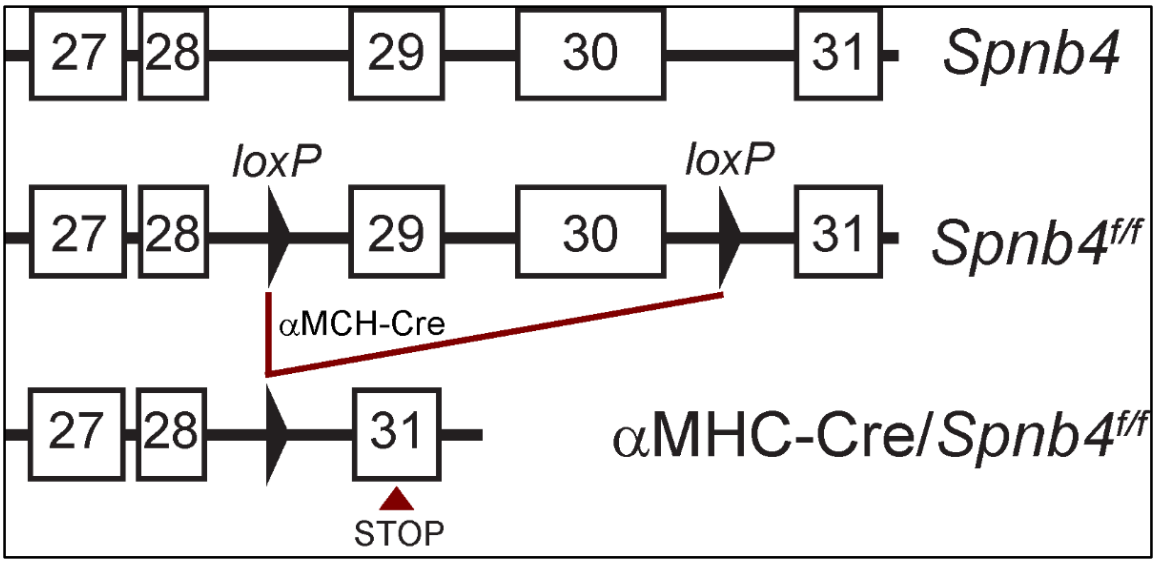

Figure 2: Schematic showing a Cre/Lox gene knockout targeting strategy used to generate the cardiac-specific β_{IV}-spectrin knockout mouse model.

Echocardiography

Cardiac function was assessed *in-vivo* by echocardiography at baseline and post Transverse Aortic Constriction (TAC) [10]. Echocardiography uses high frequency sound waves and pulse echo effect to generate live images of the heart. In this experiment, mice were anesthetized while an ultrasound transducer was placed on their chest. This technique was conducted in 8-week old male wildtype littermates, β_{IV}-spectrin cKO, and qv^{3J} to measure the ejection fraction, left ventricular chamber diameter, and diastolic left ventricular wall thickness. TAC was performed to evaluate the response to chronic adrenergic stress (pressure overload) (Figure 3). Specifically, the mice were anesthetized, intubated, and placed on a respirator. The aorta was exposed and then suture was tightened around the aorta distal to the brachiocephalic artery.

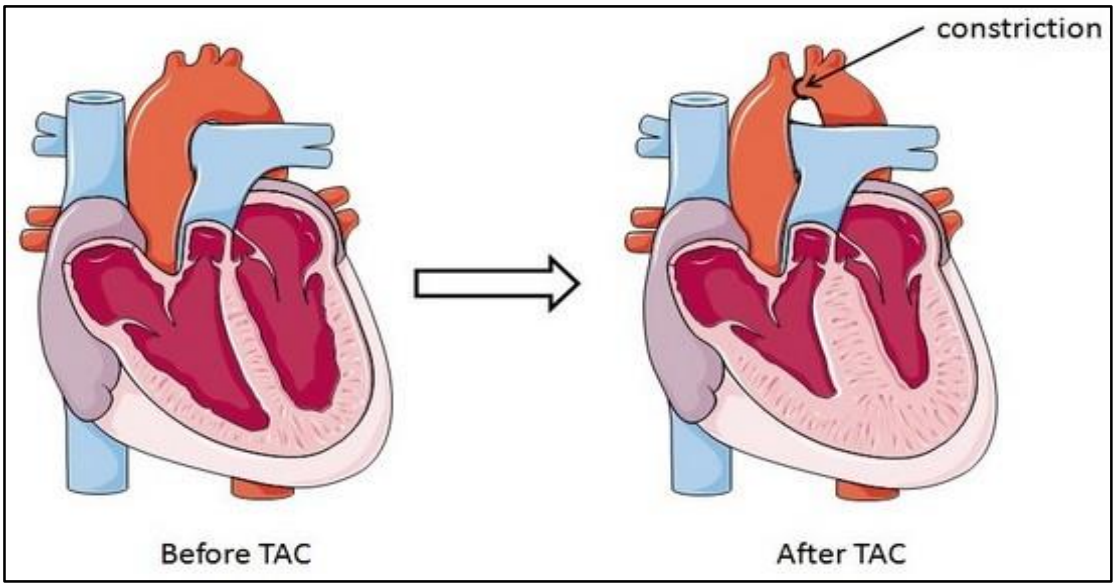

Figure 3: Schematic showing the Transverse Aortic Constriction (TAC) band on the heart to induce pressure overload conditions [11]. Suture was tightened around the aorta distal to the brachiocephalic artery

Western Blotting/Pull-Down/Co-Immunoprecipitation Assays

Western blotting experiments along with pull-down and co-immunoprecipitation (Co-IP) assays were conducted on whole heart lysates to measure relevant protein concentration and interaction/association of total and phosphorylated STAT3 and CaMKII with β_{IV}-spectrin. In western blot experiments, whole heart lysates, following quantitation by BCA assay, were loaded into 4-15% precast gels and transferred to nitrocellulose membranes. The membranes were blocked for >1 hour at room temperature in 5% milk and incubated in primary antibody overnight at 4^{o}C. Primary antibodies included β_{IV} spectrin (1:1000), ankyrin-G (1:1,000), Nav1.5 (1:500), CaMKII (1:500), TREK-1 (1:1000), STAT3 (1:1000), and GAPDH (1:5000). Secondary antibodies used were donkey anti-mouse-HRP and donkey-anti rabbit-HRP. Densitometric analysis was performed using Image Lab software to determine the presence of target proteins in the heart lysate.

Pull-down assays consisted of glutathione beads bound to a glutathione-s-transferase (GST) enzymes [9]. This technique allowed the GST enzymes to be bound to β_{IV}-spectrin proteins in heart tissue in which multiple proteins bound to the β_{IV}-spectrin could be investigated. Specifically, it allowed the separation of STAT3 bound to β_{IV}-spectrin and in turn probe whether these proteins interacted with each other to measure the level of interaction between the two using antibodies. After the glutathione beads with GST were added to the heart lysates, the samples were loaded into 4-15% precast gels and imaged to determine the level of interaction between β_{IV}-spectrin and STAT3.

Co-IP assays used target, protein-specific coupled Resin antibodies to determine the association of STAT3 with β_{IV}-spectrin in heart cell lysates of wildtype mice. A similar procedure to the pull down assay experiment was utilized to analyze the level of association of STAT3 with β_{IV}-spectrin.

Immunofluorescence

Cardiomyocytes were isolated from wildtype, β_{IV}-spectrin cKO, and *qv*^{3J} hearts. Isolated cells were fixed and permeabilized in 100% ethanol at -20ºC. Cells were blocked in 3% bovine serum albumin and 1% fetal goat serum in PBS. Cells were stained with primary antibody overnight in blocking solution. Cells were stained with secondary antibodies in blocking solution for >1 hour at room temperature. Secondary antibodies included Alexaconjugated donkey anti-mouse 488, 568 and donkey anti-rabbit 488, 568. Cells were imaged on a LSM 780 confocal microscope to study the localization of the STAT3 protein. Cardiomyocytes were imaged using identical confocal settings between genotypes. At least 20 cardiomyocytes were examined for each staining protocol.

Enzyme-linked Immunosorbent Assay (ELISA)

To measure inflammatory responses in the heart, adult ventricular cardiomyocytes were treated with an endotoxin, lipopolysaccharide (LPS), at concentrations of 50, 100, and 200 µl and cultured for 12 hours. A sandwich enzyme-linked immunosorbent assay (ELISA) experiment was used on the cardiomyocyte culture supernatant to quantitatively determine the amount of TNF-α secreted. Specifically, 100 µL of the cardiomyocyte cell culture supernatant was added to the pre-coated antibody wells, followed by an incubation period of two hours. The sample wells were aspirated, washed, added with Mouse TNF-α Conjugate, and set for an additional two hour incubation. Substrate solution followed with an acidic stop solution was added to stop the reaction of the fluorescence. The optical density was immediately determined at wavelengths 450 nm and 540nm. Based on the standard curve generated, the concentration of the TNF-α secreted from wildtype and *qv*^{3J} cardiomyocytes was determined.

Statistical Analysis

Statistical analysis was performed using the Sigma Plot software. All data is presented in the mean ± standard deviation. Statistical significance was assessed using Student's t-test for a pair-wise comparison. ANOVA was used for multiple comparisons. $P < 0.05$ was considered as significant.

Results

Echocardiography experiments at baseline revealed a reduction in cardiac function and remodeling in cardiac-specific β_{IV}-spectrin cKO (Figures 4 and 5). Ejection fraction was reduced in the β_{IV}-spectrin cKO compared to the wildtype mice (P=0.03). Interestingly, there was an increase in the cardiac function in the *qv*^{3J} mice compared to wildtype (P=0.003) or β_{IV}-spectrin cKO mice (P=0.01, Figure 5). The anterior and posterior cardiac wall thicknesses were measured during diastole. The hearts of β_{IV}-spectrin cKO mice displayed thinner ventricular walls of both the anterior and posterior cardiac chambers compared to those of wildtype or *qv*^{3J} mice (P=0.03 and 0.04, respectively, Figure 6). In contrast, wall thickening occurred in both the anterior and posterior cardiac chambers of the *qv*^{3J} mice compared to wildtype littermates. The anterior and posterior left ventricular walls were also thicker in the *qv*^{3J} mice compared to β_{IV}-spectrin cKO (P=0.04 and 0.02, respectively, Figure 6). The left ventricle of β_{IV}-spectrin cKO mice displayed significant dilation at baseline during both the systolic and diastolic phases compared to those of wildtype mice (P=0.02 and 0.03, respectively, Figure 7). In contrast, ventricular chamber during systolic and diastolic phase were smaller in the *qv*^{3J} mice indicating a mild hypertrophic response in these animals at baseline (P=0.01 and 0.04, respectively, Figure 7).

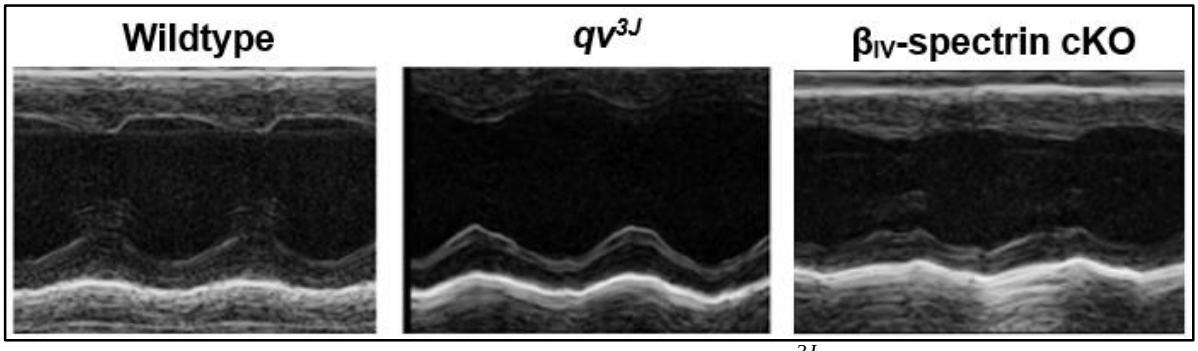

Figure 4: Representative echocardiograms from wildtype, *qv*^{3J}, and β_{IV}-spectrin cKO mice at baseline.

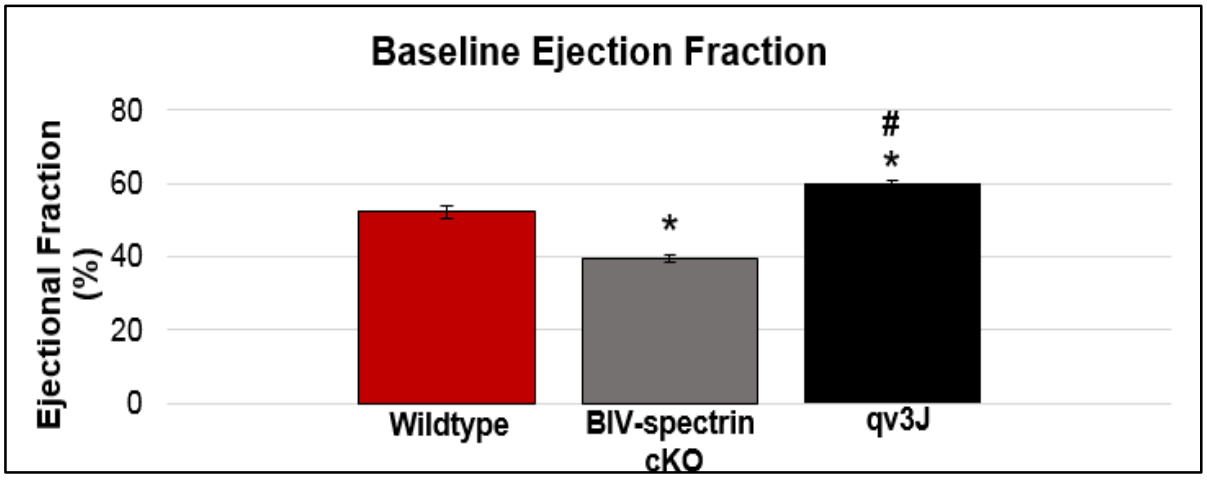

Figure 5: At baseline, 8-week old β_{IV}-spectrin cKO and *qv*^{3J} mice show increased and decreased ejection fraction, respectively compared to wildtype (control). *P <0.05 in β_{IV}-spectrin cKO and *qv*^{3J} vs. wildtype, N=5. $^{\#}$P <0.05 in *qv*^{3J} vs. β_{IV}-spectrin cKO, N=5.

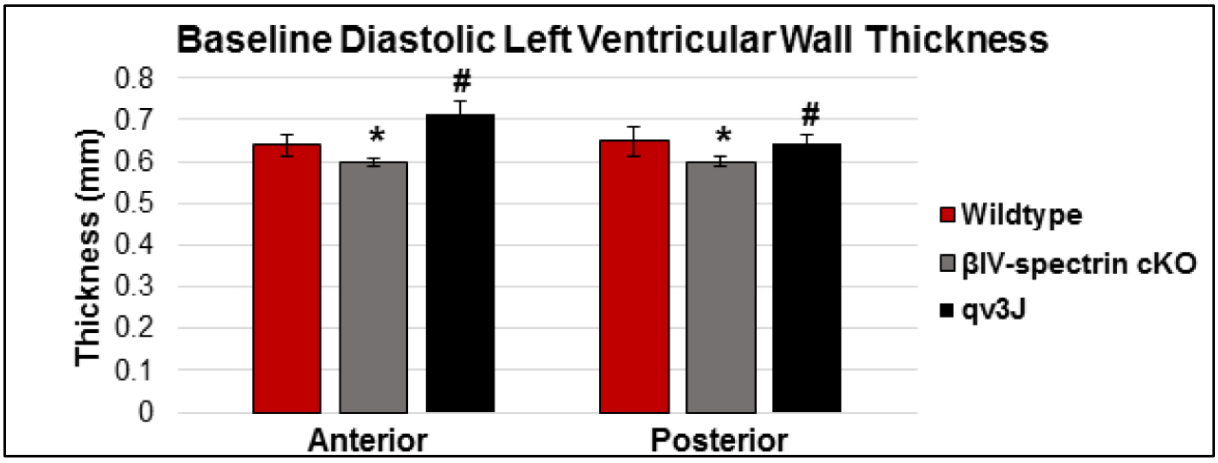

Figure 6: Echocardiography at baseline reveals that the anterior and posterior left ventricular wall thickness during diastole is thinner in β_{IV}-spectrin cKO and thicker in qv^{3J} at baseline compared to wildtype littermates (control). $^{*}P < 0.05$ in β_{IV}-spectrin cKO and qv^{3J} vs. wildtype, N=5. $^{\#}P < 0.05$ in qv^{3J} vs. β_{IV}-spectrin cKO, N=5.

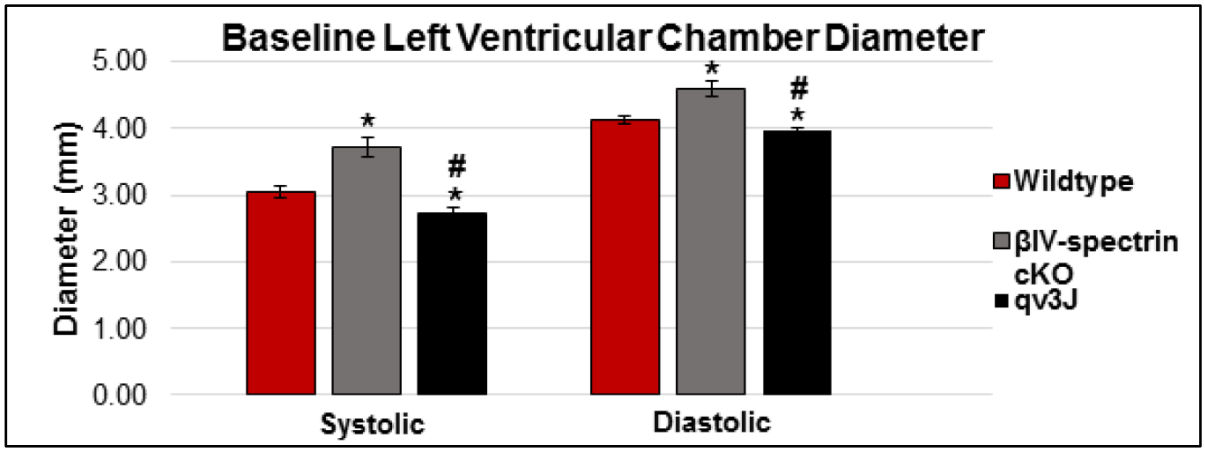

Figure 7: Echocardiography at baseline shows an increased LV chamber diameter in β_{IV}-spectrin cKO and decreased LV chamber dimeter in qv^{3J} compared to wildtype littermates (control). $^{*}P < 0.05$ in β_{IV}-spectrin cKO and qv^{3J} vs. wildtype, N=5. $^{\#}P < 0.05$ in qv^{3J} vs. β_{IV}-spectrin cKO, N=5.

Following baseline characterization of heart function in β_{IV}-spectrin cKO, *qv*^{3J}, and wildtype animals, we performed TAC (6 weeks) to evaluate their response to chronic adrenergic stress (pressure overload). These experiments revealed a more pronounced maladaptive cardiac remodeling β_{IV}-spectrin cKO compared to wildtype or *qv*^{3J} (Figure 8). There was significant decrease in the ejection fraction following 6 weeks of TAC in both wildtype and β_{IV}-spectrin cKO mice, with a more severe reduction in β_{IV}-spectrin cKO mice (P=0.002 and 0.003, respectively, Figure 9). Histological stains of β_{IV}-spectrin cKO TAC hearts displayed severe remodeling and fibrosis following the 6 weeks of TAC compared with wildtype mice and sham controls (Figure 10). In contrast *qv*^{3J} animals were resistant to maladaptive remodeling in response to TAC, showing a slight increase in ejection fraction following 6 weeks of TAC (Figures 8 and 9).

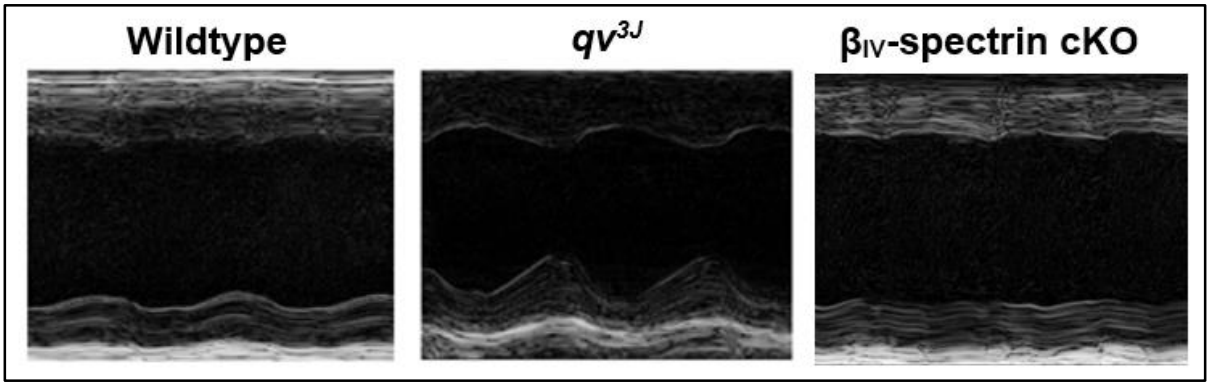

Figure 8: Representative echocardiograms from wildtype, *qv*^{3J}, and β_{IV}-spectrin cKO mice after undergoing TAC.

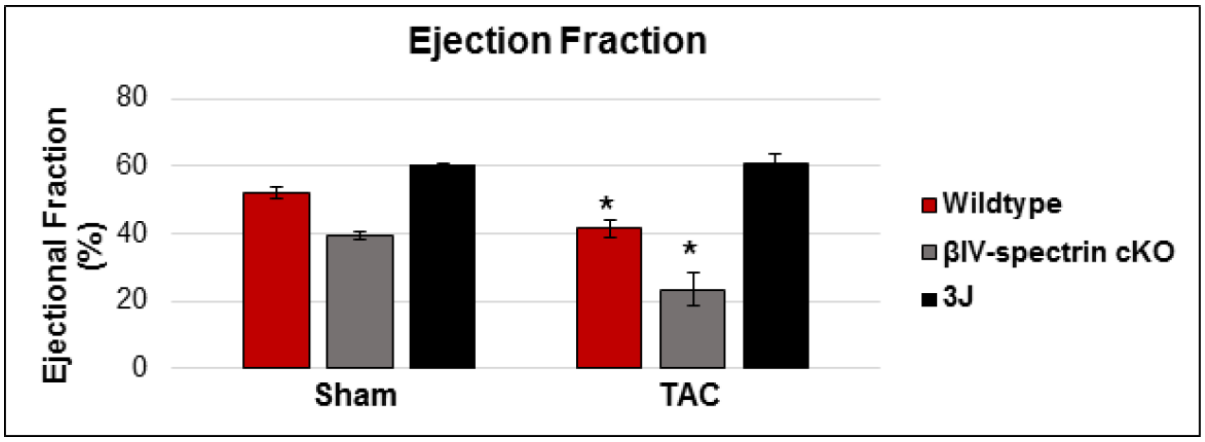

Figure 9: Echocardiography shows significant decrease in the ejection fraction in wildtype and β_{IV}-spectrin cKO mice, but not in the *qv*^{3J} model after undergoing TAC. *P <0.05 vs Sham, N=5.

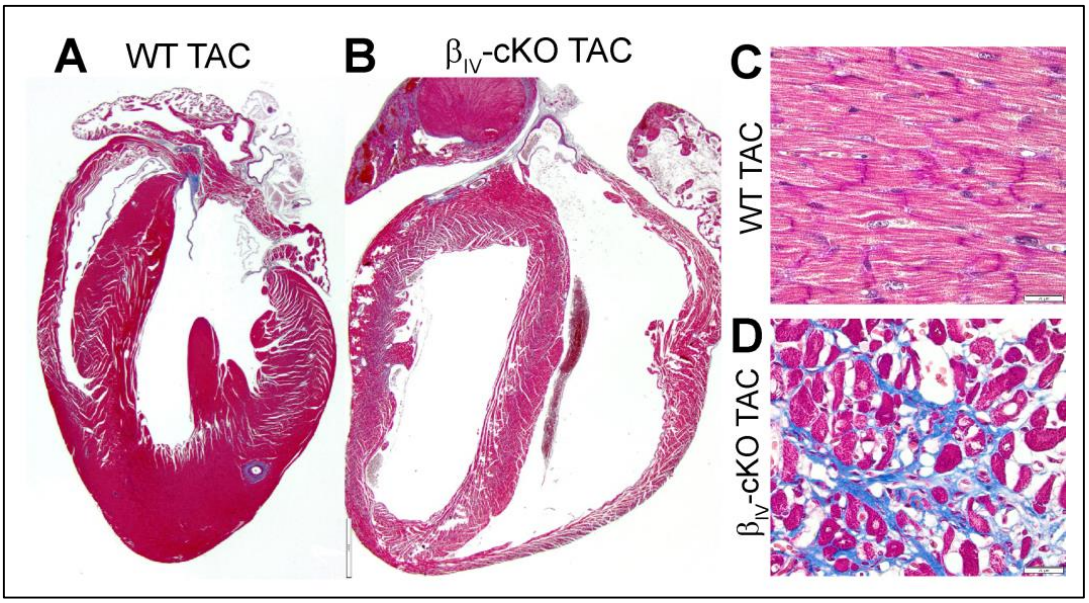

Figure 10: Histological sections of TAC β_{IV}-spectrin cKO mice show severe remodeling and fibrosis compared with wildtype TAC and Sham controls.

To determine the mechanism for observed functional differences, preliminary data on the DNA microarray of the *qv*^{3J} TAC hearts was examined (Figure 11). This technique allowed our group to measure the expression levels of variety of genes in the TAC hearts. We identified a cluster of STAT3 related genes (Figure 11B) and selective transcripts (*Nppa, Nppb and Tnfrsf*) were validated by quantitative RT-PCR (Figure 11C). Natriuretic peptide A (Nppa), natriuretic peptide B (Nppb), and tumor necrosis factor receptor superfamily (Tnfsrf) are peptides and receptors that are widely used markers for the hypertrophic response in cells or for the development of hypertrophy and heart failure related disease in animal models [12].

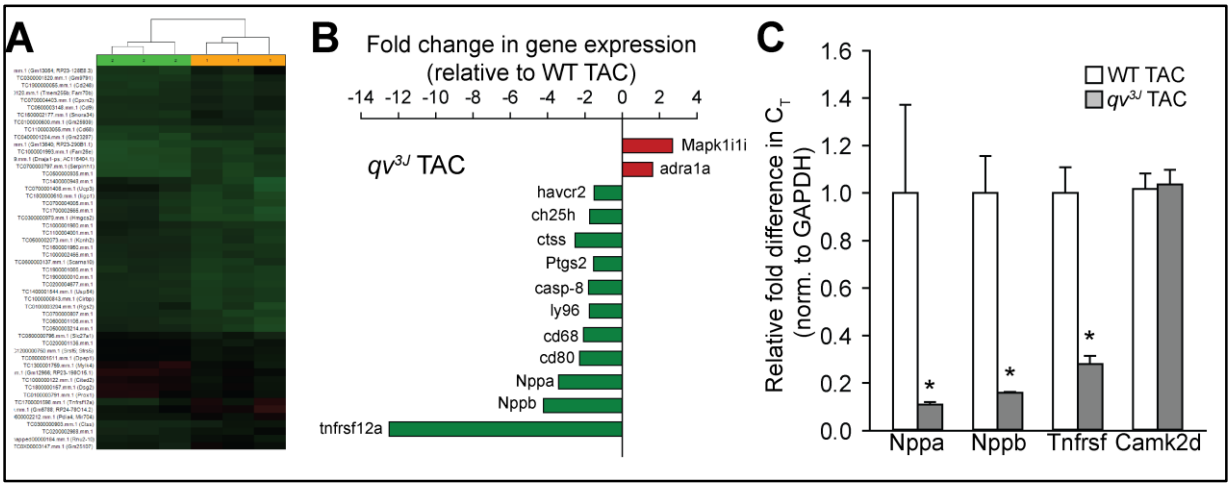

Figure 11: (A) Heat map depicting gene expression profile between WT and *qv*^{3J} TAC hearts analyzed by microarray (N=3 in triplicate /genotype). **(B)** A cluster of STAT3-related genes met the criteria of having a positive or negative fold change difference ≥ 1.5 vs. WT and P<0.05 assessed by ANOVA. **(C)** Select transcripts (*Nppa, Nppb and Tnfrsf*) were validated by quantitative RT-PCR (N=3, *P<0.05).

To test whether spectrin influences heart function via STAT3 signaling, we first analyzed STAT3 levels and localization in β_{IV}-spectrin knock-out mice. Western blot experiments demonstrated normal levels of expression of proteins Na_v 1.5, TREK-1, GAPDH whereas, Ank-G was downregulated in the β_{IV}-spectrin cKO compared to wildtype. Interestingly, the levels of total and phosphorylated STAT3 and CaMKII were not altered in the β_{IV}-spectrin cKO mice (Figure 12). Although STAT3 levels were not altered by spectrin deficiency, immunostaining and confocal microscopy showed a loss of normal localization of STAT3 in cardiac-specific β_{IV}-spectrin cKO. The wildtype cardiomyocytes displayed a prominent distribution of STAT3 at the intercalated discs (marked by N-cadherin) whereas no significant levels of STAT3 were observed at the intercalated discs of cardiomyocytes isolated from β_{IV}-spectrin cKO (Figures 13). These data suggest that loss of spectrin promotes abnormal subcellular distribution of STAT3.

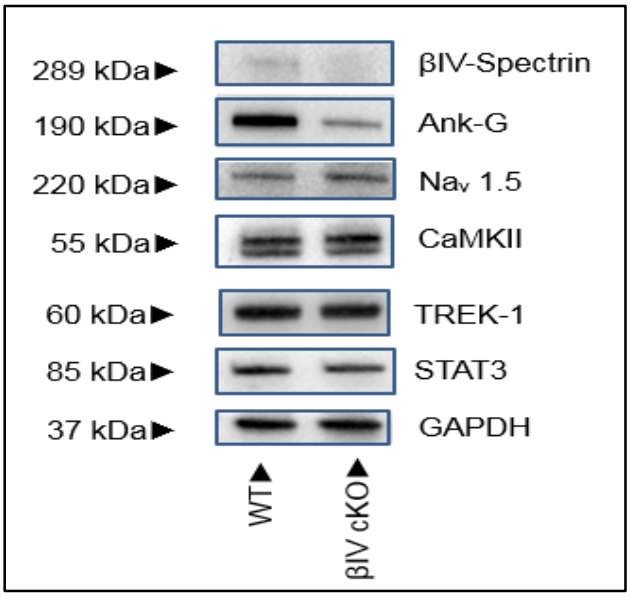

Figure 12: Western blot experiments on whole heart lysate showing levels of total and phosphorylated STAT3 and CaMKII protein concentrations in wildtype and β_{IV}-spectrin cKO mice.

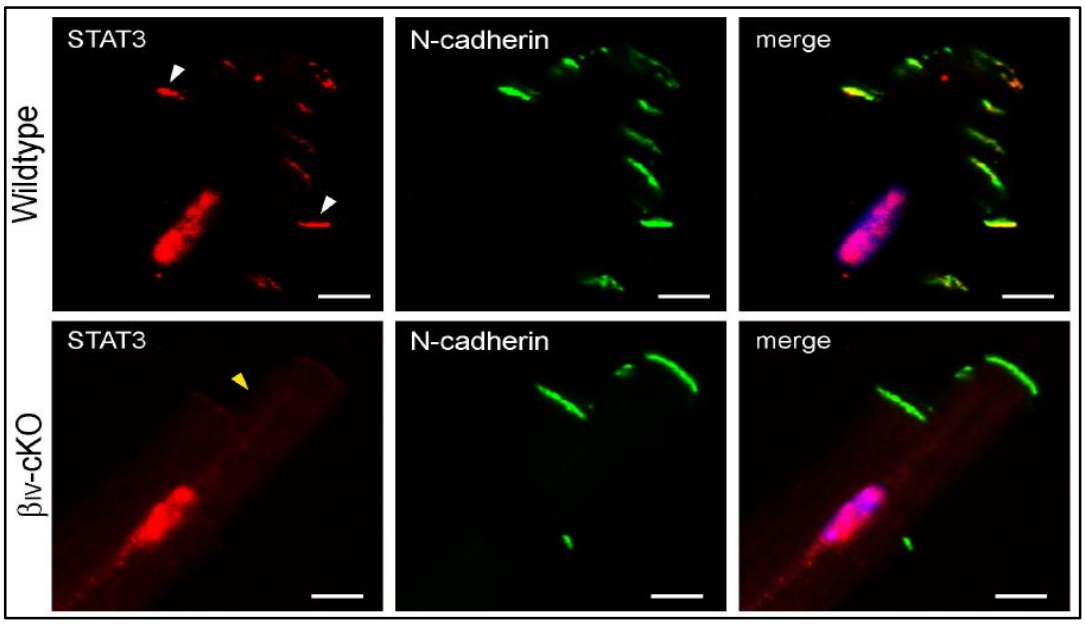

Figure 13: Confocal images of wildtype and β_{IV}-spectrin cKO cardiomyocytes show the localization of proteins through immune-staining and fluorescent labeling. Abnormal cellular location of STAT3 is observed in the β_{IV}-spectrin cKO. Scale bar = 10μm.

To determine the nature of the association between spectrin and STAT3 in heart, we performed pull-down and co-immunoprecipitation experiments. Glutathione S-Transferase (GST) protein bound to β_{IV}-spectrin from the pull-down assay suggested the interaction between the GST-β_{IV}-spectrin fusion protein (β_{IV}-WT) with STAT3 from detergent-soluble mouse heart lysates (Figure 14A). Co-IP experiments showed the association of STAT3 with β_{IV}-spectrin in detergent-soluble lysates from wildtype heart (Figure 14B).

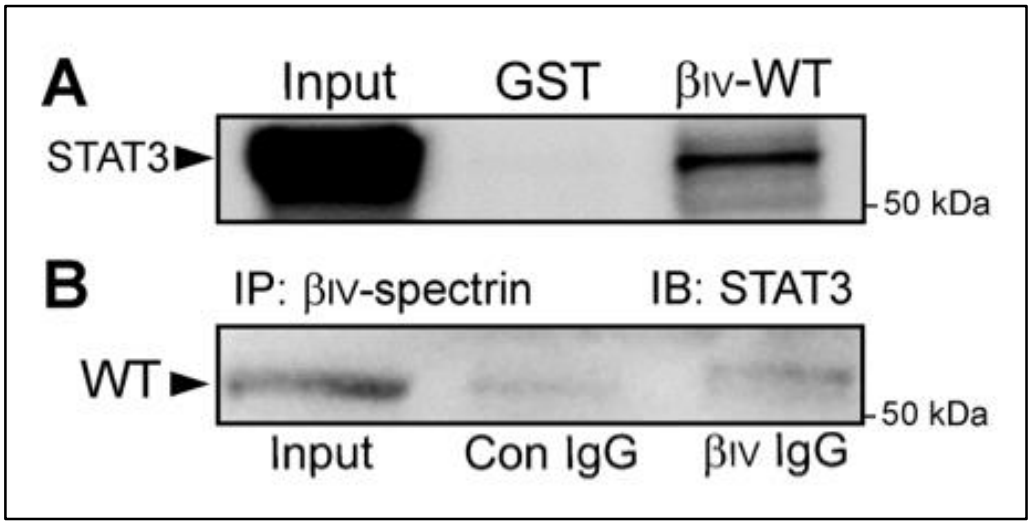

Figure 14: (A) β_{IV}-spectrin directly interacts with STAT3 in GST- protein pull-down assays from mouse heart lysate. **(B)** Co-immunoprecipitation experiments showing association of STAT3 with βIV-spectrin in detergent-soluble heart lysates from wildtype.

Finally, to determine the cellular mechanism for protection from chronic adrenergic stress in the qv^{3J} animals, we performed Enzyme-linked Immunosorbent Assay (ELISA) experiments. These experiments revealed a suppressed inflammatory reponse in qv^{3J} cardiomyocytes compared to the wildtype. After culturing the cardiomyocytes with LPS for 12 hours, there was less TNF-α secreted from the qv^{3J} cardiomyocytes compared to wildtype (Figure 15). Future experiments will investigate TNF-α seretion in β_{IV}-spectrin cKO cardiomyocyte culture supernatant.

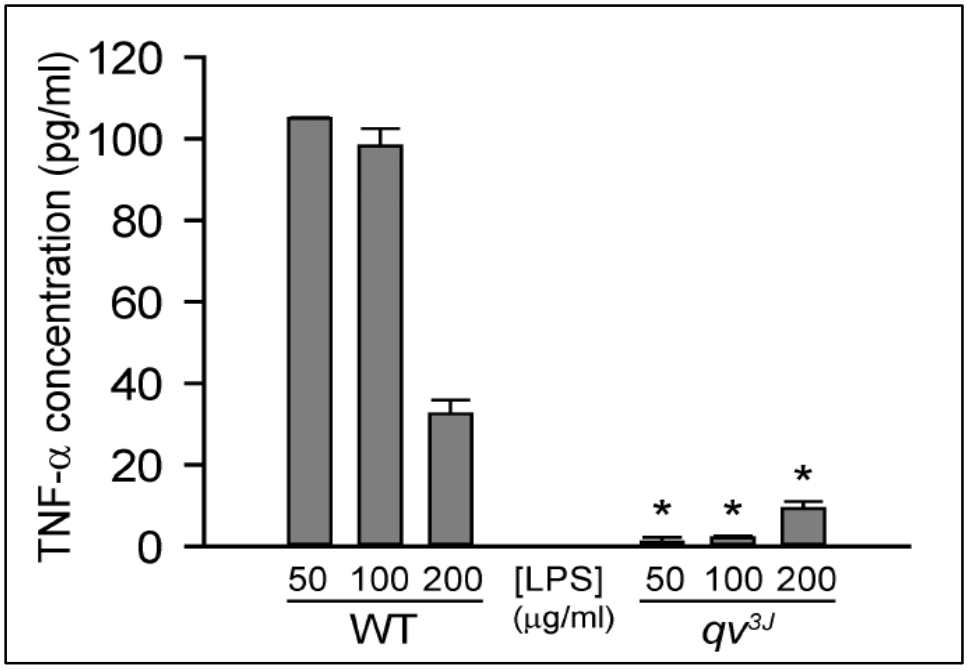

Figure 15: Adult ventricular wildtype and qv^{3J} cardiomyocytes were treated for 12 hours with LPS (50, 100, and 200 μg/ml). Levels of secreted TNF-α were assessed by ELISA as a measure of inflammatory response. $^{*}P<0.05$ vs. wildtype, N= 3.

Conclusion

This study indicates an important role for β_{IV}-spectrin in STAT3 localization and regulation in cardiomyocytes. β_{IV}-spectrin cKO mice showed decreased heart function with cardiac remodeling while qv^{3J} mice displayed normal cardiac function at baseline and post TAC compared to wildtype. Our biochemistry data revealed a novel association between β_{IV}-spectrin and STAT3 in cardiomyocytes that is essential for normal STAT3 subcellular localization at baseline and regulation in response to chronic stress. Based on this study, we propose a novel complex between β_{IV}-spectrin and STAT3 in heart (Figure 16). This β_{IV}-spectrin-based complex organizes a local signaling domain with CaMKII and STAT3 to facilitate the sensing and transduction of biomechanical stress in cardiomyocytes. Apart from localization, experimental evidence suggests that β_{IV}-spectrin also regulates STAT3 activation through CaMKII dependent phosphorylation and

thereby regulate downstream changes in gene expression in response to stress. β_{IV}-spectrin may also be a novel player that regulates cardiac inflammation by regulating the localization and phosphorylation of STAT3. This study indicates that the role of β_{IV}-spectrin is not just as an important structural component of cardiomyocytes, but also as an important mediator of intracellular signaling. Future studies will address the specific role of STAT3 in pathology associated with spectrin-deficiency. The specific binding domains responsible for β_{IV}-spectrin-STAT3 interaction will be investigated. Furthermore, in-vitro phosphorylation on STAT3 and CaMKII as well as cardiac rhythmicity will be studied. By doing so, we expect this novel complex to serve as a potential therapeutic target for cardiovascular disease patients.

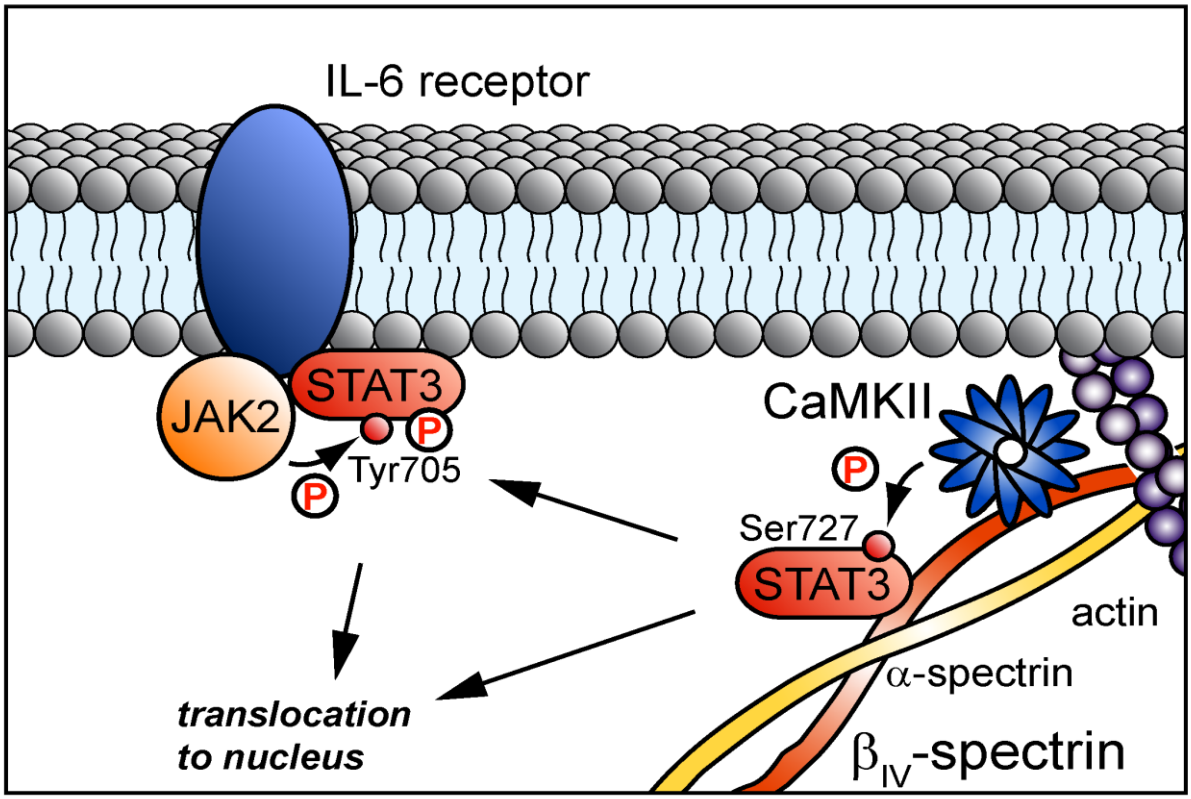

Figure 16: β_{IV}-spectrin may organize a local signaling domain with Ca^{2+}/calmodulin dependent kinase II (CaMKII) and the transcription factor STAT3 to facilitate STAT signaling in response to stress-induced receptor activation.

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